

# K-ras Oncogene Codon 12 Point Mutations in Testicular Cancer

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A significant association between *N-ras* oncogene activating point mutations and testicular cancer has recently been reported. We have studied DNA samples from the blood and fresh tumor tissues of 17 Norwegian testicular cancer patients (11 seminomas/6 nonseminomas). Point mutations in *K-ras-2* and *N-ras* exons 1 and 2 were studied by denaturing gradient gel electrophoresis (DGGE) and by oligonucleotide hybridization. No *N-ras* mutations were detected in these tumor samples, but two *K-ras-2* exon 1 mutations were found in two of the seminoma tumors (stage I and II tumors) using the DGGE technique. The mutations were confirmed by dot blotting and oligonucleotide hybridization and identified as a G→T and a G→A point mutation in *K-ras-2* codon 12, leading to a valine and a serine substitution, respectively. All the white blood cell DNAs were negative. As a positive control for DGGE screening, we ran two plasmid constructs carrying human *N-ras* exon 2 sequences with mutations. To study the role of *ras* gene activation in testicular cancer, a larger tumor sample population will be investigated.

## Introduction

Testicular cancer is one of the most frequent cancers among young men. A higher risk of testicular cancer has been associated with certain subpopulations, e.g., men of higher socioeconomic status and some occupational groups such as paper and printing workers, professionals, and administrators (1). Other anatomic, genetic, and environmental risk factors also exist.

The most common activated oncogenes in human cancers are *ras* oncogenes (2). This oncogene family contains three closely related cellular genes: *K-ras-2*, *H-ras-1*, and *N-ras* (3). The *ras* genes are almost exclusively activated by single base-pair substitutions (4), most frequently in codons 12, 13, or 61. Different members of the *ras* oncogene family are activated in various types of cancer (2,5). In testicular cancer, *N-ras* point mutations have frequently been detected in codons 12 and 61 (6,7). However, Mulder et al. (8) also reported *K-ras-2* codon 12 base substitutions in a few of the cases.

We studied *K-ras-2* and *N-ras* mutations in samples from 17 Norwegian testicular cancer patients by denaturing gradient gel electrophoresis (DGGE) and by dot blotting and

oligonucleotide hybridization (8,9). The combination of these techniques has turned out to be suitable for rapidly screening for mutations in *ras* oncogenes (10). DGGE is based on the ability of DNA to melt in different sequence-dependent domains. Theoretically, one base pair substitution in a lower melting domain is enough to change the melting behavior of a DNA sequence (11-13).

## Materials and Methods

DNA samples from the peripheral blood and fresh tumor tissue of 17 Norwegian testicular cancer patients were studied. The patients were 22-53 years old at the time of diagnosis, and they are a subgroup of cases already described by Lothe et al. (14). According to a light microscopic diagnosis, 11 of the patients had a unilateral seminoma and 6 had a unilateral nonseminoma.

*K-ras-2* and *N-ras* exons 1 and 2 were amplified separately by the polymerase chain reaction (PCR) using AmpliTaq-DNA polymerase (Cetus). In each of the PCRs, one of the primers had a GC-rich oligonucleotide sequence of 40 base pairs (a GC-clamp), which was introduced at one end of the amplified sequences (15,16). Sequences for the primers are given elsewhere (Ridanpää and Husgafvel-Pursiainen, submitted.). The length of the PCR products varied between 150 and 175 base-pairs.

Aliquots of the PCR products were analyzed in denaturing gradient gel electrophoresis (DGGE). Perpendicular and parallel gels were run using 7.5% polyacrylamide gels containing a linear gradient of chemical denaturants. In

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the perpendicular gels, the gradient was 0–50% and in the parallel gels it was 25–40% (100% denaturant = 7 M urea and 40% formamide). All gels were run at 60°C in a bath of Tris-acetate-EDTA (TAE) buffer at pH 8.0. The gels were stained with ethidium bromide in distilled water before photographing under UV light.

Two plasmids carrying a single base-pair substitution in human *N-ras* codon 61 (CAA→AAA and CAA→CAC) were constructed (16). After *in vitro* amplification, the *N-ras* mutant sequences were run in DGGE as a control for the separation of the mutant bands from the wild-type ones. Specimens from the same PCR products were dot blotted on nylon membranes (Hybond) with a set of positive control DNAs for activating point mutations in *K-ras*-2 and *N-ras* in codons 12, 13, and 61 (Clontech Laboratories). <sup>32</sup>P-end-labeled oligonucleotide probing, hybridization, and washing procedures were done according to the manufacturer's recommendation. Autoradiography films (Amersham) were exposed at –70°C using intensifying screens.

## Results

Among 11 seminomas, there were two cases (stage I and II) that were observed to carry a *K-ras*-2 exon 1 mutation by DGGE. A clear separation between the homoduplex wild-type band and the mutant homoduplex bands were obtained. By oligonucleotide hybridization, these two mutations were identified to be a G→T transversion (glycine to valine substitution) and a G→A transition (serine substituted for glycine) in *K-ras*-2 codon 12 (Fig. 1). The rest of the seminoma samples and all of the non-seminoma samples showed neither *N-ras* nor other *K-ras*-2 mutations (Table 1).

The two *in vitro* amplified *N-ras* sequences each carrying a single base-pair substitution in codon 61 (CAA→AAA, CAA→CAC) gave a good separation in the denaturing gradient gels (Fig. 2). In tumor samples, in addition to the well-separated homoduplex bands, heteroduplexes were seen on the denaturing gradient gels. The heteroduplex

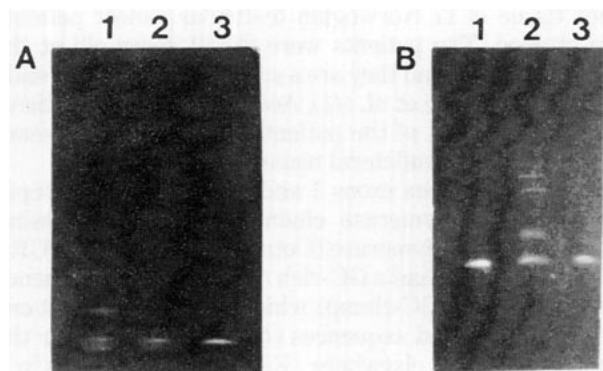


FIGURE 1. Detection of point mutations in *K-ras*-2 exon 1 by denaturing gradient gel electrophoresis. Testicular tumor samples on a parallel gel (denaturing concentration 25–40%). (A) Lane 1 shows a mutation (codon 12 valine, GGT→GTT). (B) Lane 2 shows a mutation (codon 12 serine, GGT→AGT). In both figures the other lanes represent wild-type.

Table 1. The set of testicular tumor samples studies.<sup>a</sup>

Histological diagnosis	No. of tumors	Mutations in <i>K-ras</i> -2 exon 1 (codon 12)	Amino acid substitution
Seminoma	11	2	Serine-12 Valine-12
Nonseminoma			
Embryonal carcinoma	3	None	
Endodermal sinus tumor	2	None	
Mature teratoma	1	None	

<sup>a</sup>No *K-ras*-2 exon 2 (codon 61) or *N-ras* mutations were detected.

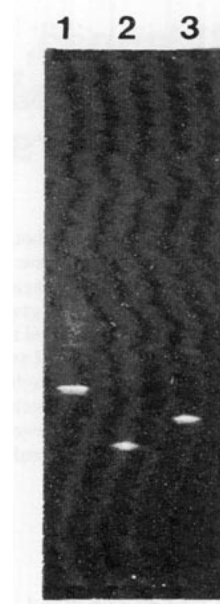


FIGURE 2. Detection of point mutations in *N-ras* exon 2 in a parallel gel electrophoresis (denaturing gradient 25–40%). (Lane 1) Positive control sequence carrying codon 61 lysine (AAA); (Lane 2) positive control sequence carrying codon 61 histidine-2 (CAC); (Lane 3) *N-ras* exon 2 codon 61 wild-type (CAA).

bands of the valine mutant displayed greater separation than the heteroduplex bands of the serine mutant, thus facilitating the identification of the mutants.

## Discussion

*N-ras* point mutations in codons 12 and 61 have been detected in 4/14 (6) and 20/31 (7) tumors of testicular cancer. In addition, Mulder et al. (6) reported two *K-ras*-2 codon 12 mutations in a group of 14 testicular cancers. In the present study, the only *ras* gene mutations found in the fresh testicular tumor samples from 17 patients were those in *K-ras*-2 codon 12. Both of the *K-ras*-2 mutations were detected in seminoma tumors, one being a stage I and the other a stage II tumor. This is in accordance with the reported higher incidence of *ras* gene mutations in seminoma as compared to nonseminoma tumors (7).

Compared to our findings in lung cancer, using a similar approach (10), the observed *K-ras*-2 mutation frequency in

testicular cancer seems to be much lower. In lung adenocarcinoma, *ras* gene activation seems to occur in early stages of tumor development and has been associated with poor prognosis (17). Most of the mutations found in lung adenocarcinomas are reported to be G→T transversions in K-*ras*-2 (10,15) and the mutation type appears to be associated with smoking (18). In the literature, however, no such qualitative predominance in the types of base-pair substitutions in K-*ras*-2 or N-*ras* genes has been found in testicular cancer (6,7). The amount of cancer, however, is too small to draw any firm conclusions about the nature and the frequency of the *ras* oncogene mutations in testicular cancer. To further elucidate the role and type of *ras* gene mutations in testicular cancer, we are now in the process of cases investigating a larger set of tumor samples.

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